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TITLE: Targeting LSD1 Epigenetic Signature in Castration-Recurrent Prostate Cancer

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14. ABSTRACT

Prostate cancer (PCa) is the second most common cause of cancer death in men in the US. PCa is initially driven by circulating androgens that activate AR and its downstream targets. AR inhibition is a primary therapeutic intervention but patients often relapse and develop castration recurrent PCa (CRPCa). This is often clinically lethal. Here we describe a potential therapeutic approach for patients that develop CRPCa. We demonstrated that targeting AR and its coregulator LSD1 lead to additive antiproliferative effect *in-vitro* and potentially reduces CRPCa progression *in-vivo*. Ongoing studies aim to describe the genome-wide signature of AR and LSD1 in androgen responsive and CRPCa cell lines, in addition to cell lines with DHT-activated AR. Validation of this approach in our pre-clinical studies can have a beneficial impact on PCa patients since it defines a novel therapeutic regiment that relies on already clinically available drugs.

15. SUBJECT TERMS

AR(androgen receptor), LSD1(lysine specific demethylase 1A), PCa(prostate cancer), CRPCa(castration recurrent PCa), DHT(dihydroxy testosterone)

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INTRODUCTION:

Prostate cancer (PCa) is the most commonly diagnosed cancer in males in the US and second most common cause of cancer death. Clinical management of PCa includes androgen deprivation therapy (ADT) whose aim is to reduce the levels of circulating androgens. This approach often leads to tumor shrinkage and disease remission, however, about 30% of patients who underwent ADT will recur and PCa will recur under castration condition. This clinical manifestation, named castration recurrent (PCa), is the most aggressive and often lethal. The androgen receptor (AR) is activated by circulating androgens, it's a main driver in PCa progression and its inhibition leads to prostate shrinkage. Numerous molecules have been developed to target the AR, the most common being Bicalutamide (Casodex), but patients often develop resistance and more aggressive disease, named castration recurrent PCa (CRPCa). The AR activity is modulated by a cohort of regulatory proteins that, depending from the genomic context, can promote or repress its activity. The lysine specific demethylase 1A (LSD1) is a key coregulator for AR, shown to mediate AR transcriptional activity and, subsequently, mediate AR downstream signaling. LSD1 structure closely resembles a monoamino oxidase (MAO) enzyme and therefore can be pharmacologically targeted by tranylcypromine (TCP), a MAO-inhibitor commonly used for psychiatric disorders. TCP has antiproliferative effect on a number of cancer cell lines and it has been suggested as potential therapeutic agent. Here we propose a combinatorial therapeutic approach that inhibits AR and LSD1 activity in order to reduce the recurrence rate of CRPCa. The approach includes *in-vivo*, *in-vitro* and *in-silico* analysis to profile LSD1 and AR signature and its implications for therapy.

KEYWORDS:

ADT - androgen deprivation therapy
AI - androgen independent
AR - androgen receptor
AS - androgen sensitive
Bic - bicalutamide
ChIP-Seq - Chromatin Immunoprecipitation followed by DNA sequencing
CRPCa - Castration recurrent prostate cancer
CWR22 - androgen sensitive prostate cancer cell line
CWR22-RV1 - castration recurrent prostate cancer cell line, androgen independent
DHT - dehydroxy testosterone
LNCaP - androgen sensitive prostate cancer cell line
LNCAP-C42 - castration recurrent prostate cancer cell line, androgen independent
LSD1 - lysine specific demethylase 1A
NR – nuclear receptors
PCa - prostate cancer
RNA-Seq - RNA sequencing
TCP - tranylcypromine

ACCOMPLISHMENTS:

What were the major goals of the project?

The goals (aims) stated in the SOW were the following:

1) To define the genome wide binding sites of LSD1 in ASPCa and CRPCa
2) To define the functional consequences of LSD1 binding to its target genes in ASPC and CRPCa.

3) To evaluate the chemopreventive and therapeutic effect of TCP and bicalutamide on the development of CRPCa in vivo.

During the first 12 months:

- goal 1) is 60% complete,
- goal 2) includes only two points, one is complete and the second one depends on full completion of goal 1),
- goal 3) is 100% completed

What was accomplished under these goals?

1) Cell lines LNCaP, LNCaP-c42, CWR22-Rv1, CWR22PC, VCaP and VCaP-CR cells were thawed from liquid nitrogen and expanded. Several weeks were spent to passage and grow cells in order to be able to freeze down aliquots, should they be needed as backup in case of problems such as bacterial contamination or micoplasm infection came up.

Next, to better describe the LSD1 genomic signature in the AR context, it is important to consider AR dynamicity since LSD1 not only modulates basal AR activity, but also agonit-bound AR. To capture this picture we decided to prepare LNCaP and LNCaP-C42 cells for LSD1 ChIP-Seq by treating them for 1 hour with 10nM DHT (AR natural agonist). This will provide the data described by the proposal, but in addition will show how LSD1 relocates across genes that are actively targeted by AR upon androgen stimulation. Treatments were performed in biological duplicates, samples were collected, sonicated, fragments purified and correct chromatin fragment sizes was checked by running an aliquot of the sample on an agarose gel. Only samples with chromatin fragments from 100-500 were used for the pull-down with LSD1 antibody. CWR22-Rv1 and VCaP cells were also sent to the core facility with LNCaP and LNCaP-C42. Sequencing libraries have been prepared and samples are currently queued at the Genomic Core facility at Roswell Park and results will be available shortly for the final analysis.

2) Cell lines needed are ready to be used for validation and are currently being passaged and frozen down to ensure that we have enough usable aliquots in case we would need to thaw a new batch while the experiments are running.

3) *in-vivo* experiments aimed to evaluate the pre-clinical significance of the combined treatment on the onset of CRPCa. CWR22 cells were subcutaneously injected in 10 nude mice in order to grow enough cells for the experiment. Tumors were resected, washed and digested in trypsin before cell counting. Cells in a matrigel suspension were then injected subcutaneously in 200 mice (10^6 cells/mouse). Testosterone pellets were prepared in order to deliver 12.5mg of hormone per mouse and were implanted subcutaneously. Mice were randomized into four groups: control, bicalutamide, TCP, bicalutamide + TCP. Once tumor reached 200mm^3, pellets were removed in order to mimic clinical ADT, and mice were treated daily (5 days on, 2 days off) with 10mg/kg of bicalutamide, TCP or both for 9 weeks. Drugs were delivered intraperitoneally and tumor volume was monitored weekly via caliper measurements. Tumors are collected at time of death for future immunohistochemical studies together with blood for serum PSA measurements.

Week 0 corresponds to pellet removal and start of treatment. At week 30 roughly 50 to 70% of the mice, depending from the group, are still in the study (**fig 1**).

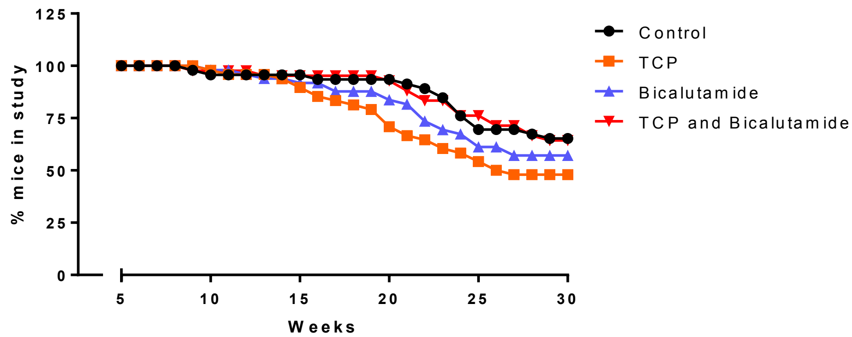


Figure 1 - *Number of mice still in the study. Mice are euthanized when tumor size reaches 2cm in any dimension or when signs of toxicity appear. The y axis indicates the percentage of remaining mice compared to the starting number at week 5. Mice that died before week 5 were not taken into consideration.*

Figure 2 shows the changes in tumor volume across all mice. Weekly measurements suggest a trend in the TCP+Bic group as being delayed when compared to the other 3 groups. Furthermore, mice treated with both agents developed fewer metastases than the other mice (**table 1**)

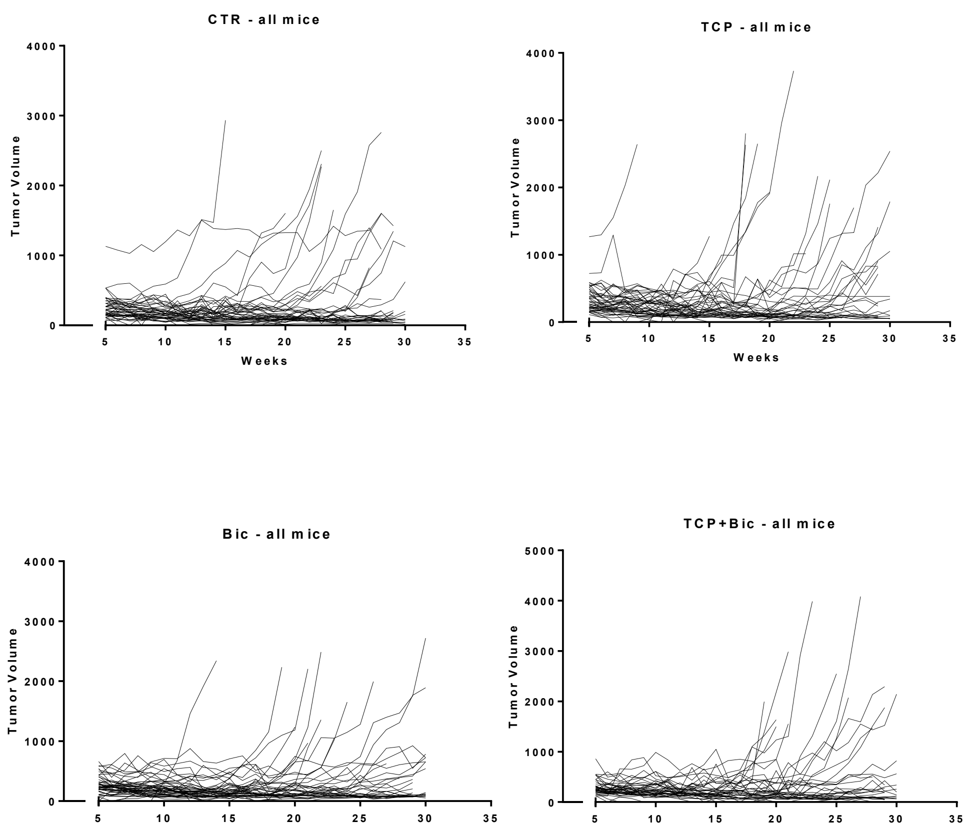


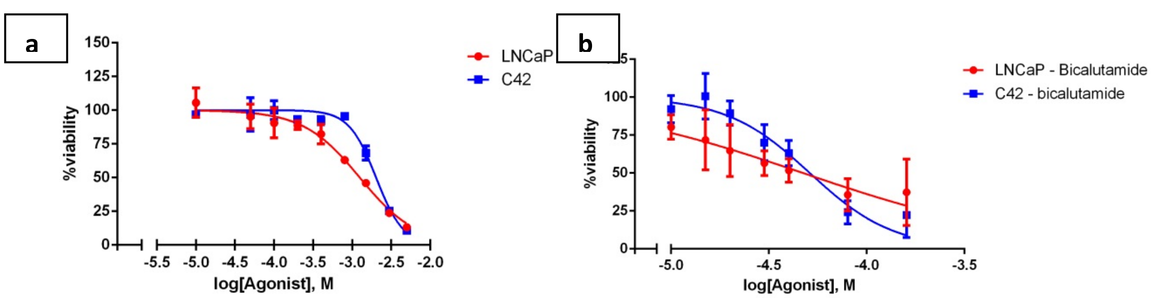
Figure 2 - *Tumor volume across all samples. Volume was calculated by measuring the longest side times the square of the shortest size and plotted on the y axis while the x axis represent the week number from the start of the treatment.*

Group	# mice with mets
control	9
TCP	15
Bic	11
Bic+TCP	7

Table 1 - *Count of the mice that developed metastasis since the beginning of the study.*

Mice will be measured weekly until the end of the project and, in case of premature exit from the study, organs and blood will be collected for immunohistochemical analysis and PSA measurements, respectively.

other achievements) Since the main goal of this grant is to describe and evaluate the potential of an alternative therapeutic approach using TCP and bicalutamide in combination, we sought to confirm that we were using the correct system to study AR and LSD1 *in-vitro* by showing that the combinatorial treatment has an additive antiproliferative *in-vitro* effect before carrying on with any molecular characterization. The additive effect of TCP and Bicalutamide was evaluated in both androgen sensitive and castration recurrent conditions by using LNCaP and LNCaP-C42 cells. Furthermore, those are the cells that will be used for validating ChIP data and for gene expression analysis. Treatment with increasing concentrations of bicalutamide and TCP show a dose-depended response to the ligands (**fig 3a-b**). Furthermore, 96 hours combined exposure with IC50s and IC25s of both drugs showed significant additive effect in inhibiting cell viability (**fig 3c-f**).



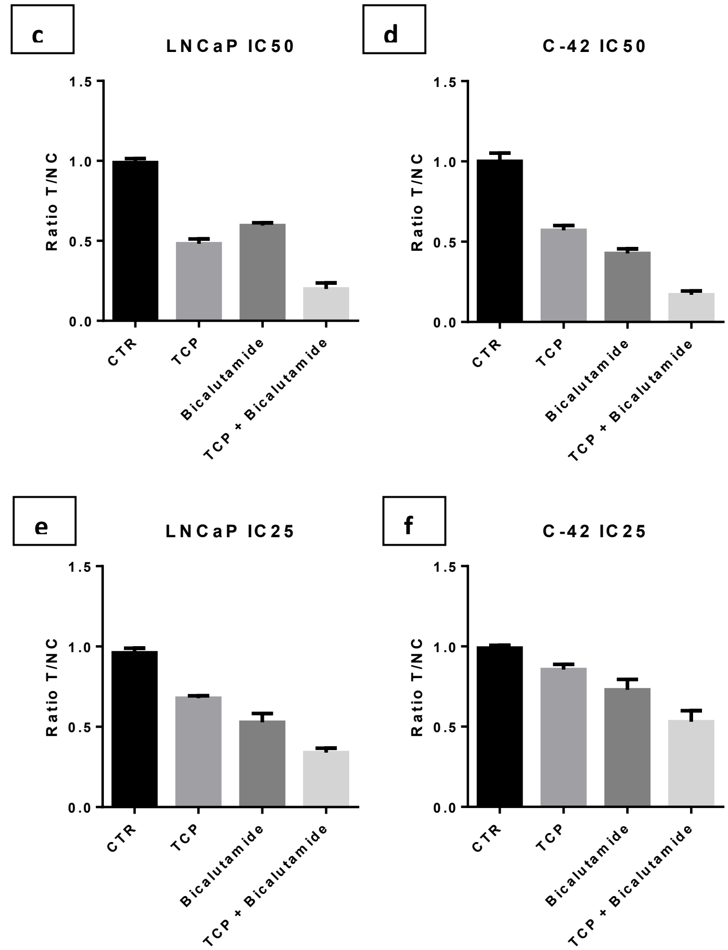


Figure 3 - *In-vitro antiproliferative effect of bicalutamide and TCP. 2000 cells/well were seeded in a 96 well plate and different concentrations of TCP and bicalutamide were used to identify the IC50 and IC25 per each cell line (**a, b**). Combinatorial treatment with IC50s and IC25s of both TCP and bicalutamide resulted in additive inhibition of cell viability in both LNCaP and LNCaP-C42 cells (**c-f**).*

These results support the goals of the project by showing that *in-vitro* the combination of TCP and bicalutamide has an additive effect on inhibiting prostate cancer cell proliferation with low or no toxicity detected.

Goal's 1) progresses are consistent with the main goal of the project of describing the LSD1-AR genomic signature. The extra time spent in properly preparing the cell lines, in considering AR dynamicity and preparing experiments will offer a deeper understanding of AR and LSD1 interactions in both androgen sensitive and castration recurrent settings, as initially described in the project. Furthermore, the data obtained from DHT treatment will be an extremely valuable addition to the expected results since we will be able to describe LSD1 dynamic response upon AR activation and its differential androgen-driven recruitment across the genome. The remaining steps for completion of goal 1) are technically trivial and allow for parallel experiments to be completed and no major delays are expected for the completion of the project.
Goal's 2) progresses are in line with the expected timeline and primer design and purchase will immediately follow the analysis of the data from goal 1).
Goal's 3) achievements were fully met and no delays are expected in completing it by the end of the study.

What opportunities for training and professional development has the project provided?
Goal 1) Working with all of those cell lines allowed me to learn about ChIP-Seq sample preparation and about the strict necessity of having the correct chromatin fragmentation. This is a key step that could compromise the sequencing reaction (and the final results) if not addressed properly. Furthermore, my co-mentor Dr. Liu introduced me to the file format that I will be working with as soon as the data are available. He explained me how to properly use .fastq files to analyze ChIP-Seq data and retrieve significant peaks and targeted genes coordinates. This will allow me to promptly extract the results and map the genes targeted by LSD1. Furthermore, I was exposed to other publicly available ChIP-Seq datasets and Dr. Liu suggested bioinformatics research papers that would help me grasp the nuances of next generation sequencing data analysis. These progresses are in line with the training goals of my project.

Goal 3) These progresses, and results, are consistent with the project goals. The training goals are also being met, since my mentor Dr. Foster trained me in the *in-vivo* aspect of the work since I learned to administer drugs to mice, how look for signs of sickness and toxicity and how to perform most of the basic tasks required to handle nude mice for this part of the project. Furthermore, I discussed the preclinical implications of those preliminary results with my co-mentor Dr. Pili, who helped me understand the translational components and the clinical aspect, and interpretation, of this project. I will continue to closely work with Dr. Foster and Dr. Pili until the project ends, in order to grasp all the details needed to fully interpret the translational importance of this study.

In November 2013 I also attended the Society for Basic Urologic Research (SBUR) conference in Nashville (TN) that allowed me to interact with worldwide scientists and discuss my project. Being a urology-focused meeting, I was able to talk to numerous experts in PCa that gave me feedback and helped me develop a more complete project in order to meet the stated goals.

How were the results disseminated to communities of interest?
I presented the obtained results to various meetings and seminars at Roswell Park, allowing me to get useful feedback from both clinicians and researchers.
Furthermore, a brief description of my project was published on my institution's website which is publicly accessible. For the benefit of the lay reader, the description was very concise, using broad terms.

What do you plan to do during the next reporting period to accomplish the goals?
During the next reporting period I plan on completing the study by dividing the work in two main blocks: 1) analysis of ChIP-Seq data and validation and 2) completion of the *in-vivo* study.
For 1) I will work closely with Dr. Liu to make sure that the statistical analysis is make correctly and can be reproduced by other users (in case the raw data will be publicly available after publication). I will also work with Dr. Liu to integrate publicly available data for AR ChIP-Seq in order to describe the cistrome regulated by LSD1 and AR in PCa.
For 2) I will closely work with Dr. Foster for completion of the *in-vivo* study since her knowledge and expertise in animal models is unique and fundamental for my learning curve. I will also constantly report *in-vitro* and *in-vivo* data to Dr. Pili so that I can discuss with him the translational implication of the project and, if needed, improve it.

IMPACT

The results obtained so far are extremely encouraging since they support the initial hypothesis that AR and LSD1 are important drivers of PCa progression. The *in-vitro* data are in accordance with the initial *in-vivo* results suggesting that this pharmacological approach is consistently acting to suppress PCa cell growth. The impact of these findings lays on the possibility of having an alternative therapeutic approach for patients that develop CRPCa and do not respond well to the initial bicalutamide regiment (ADT)

What was the impact on the development of the principal discipline(s) of the project?

This approach demonstrates the importance of coregulatory proteins in the nuclear receptors signaling network. Only a few studies aimed to pharmacologically target nuclear receptors and their coregulators in order to propose a new therapeutic approach. This underlies how a single-agent therapy is often not sufficient to fight aggressive cancers. Resistance to therapy is a key problem in the clinic and a combinatorial approach that targets cancer under a different, but parallel, pathway can lead to key development that ultimately benefit cancer patients.

What was the impact on other disciplines?

Nothing to report

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

CHANGES/PROBLEMS:

Changes in approach and reasons for change

No changes were made to the project as described in the original application. Additional data were generated to support the hypothesis that the combinatorial treatment had a significant impact on PCa cell proliferation. The generation of those data supported the proposed experiments in LNCaP and LNCaP-C42 since they demonstrated to be the perfect model where to study AR and LSD1 combined action.

Because of those positive findings, further experiments were done by treating LNCaP and LNCaP-C42 with 10nM DHT before harvesting cells for LDS1 ChIP-Seq. These additional experiments will not change the approach that was originally described, but instead will provide further support to the hypothesis that LSD1 and AR act coordinately, and dynamically, across the genome to define a signature that drives PCa progression.

Actual or anticipated problems or delays and actions or plans to resolve them

No additional delays are expected and the study is expected to be complete in accordance with the study's deadlines

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to report

Significant changes in use or care of human subjects
Nothing to report

Significant changes in use or care of vertebrate animals.
Nothing to report

Significant changes in use of biohazards and/or select agents
Nothing to report

PRODUCTS:
Nothing to report

Publications, conference papers, and presentations
Nothing to report

Journal publications:
Nothing to report

Books or other non-periodical, one-time publications:
Nothing to report

Other publications, conference papers, and presentations:
Nothing to report

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Sebastiano Battaglia*
Project Role:	*Post-doc*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*Full time*
Contribution to Project:	*PI*
Funding Support:	*DOD postdoctoral training award*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to report

What other organizations were involved as partners?
Nothing to report

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:
Nothing to report

QUAD CHARTS
Nothing to report

APPENDICES:

Nothing to report